

AD _____

Award Number: DAMD17-98-1-8084

TITLE: FC-gamma Receptor-targeted Immunization for Breast Cancer

PRINCIPAL INVESTIGATOR: Louis M. Weiner, M.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

REPORT DATE: May 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020123 089

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)			2. REPORT DATE May 2001		3. REPORT TYPE AND DATES COVERED Final (15 Apr 98 - 14 Apr 01)	
4. TITLE AND SUBTITLE Fc-gamma Receptor-targeted Immunization for Breast Cancer			5. FUNDING NUMBERS DAMD17-98-1-8084			
6. AUTHOR(S) Louis M. Weiner, M.D.						
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Fox Chase Cancer Center Philadelphia, Pennsylvania 19111 E-Mail: LM_Weiner@fccc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER			
11. Supplementary Notes						
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited						12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words)						
The objective of this research project is to test the hypothesis that immune responses to a cancer antigen that is commonly overexpressed in breast cancer can be selectively enhanced and lead to protection against such tumors. This hypothesis of Fc γ receptor-targeted immunization is being tested through the construction of fusion proteins containing a fragment of the extracellular domain of the HER2/neu antigen and an antibody Fc γ domain. The resulting fusion protein should target an immunogenic and tumor growth modulatory epitope of HER2/neu to antigen-presenting cells via Fc γ receptors.						
14. SUBJECT TERMS Breast Cancer						15. NUMBER OF PAGES 10
						16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited			

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	9
References.....	10
Appendices.....	N/A

FOREWORD

The objective of this research project is to test the hypothesis that immune responses to a cancer antigen that is commonly overexpressed in breast cancer can be selectively enhanced and lead to protection against such tumors. This hypothesis of Fc γ receptor-targeted immunization is being tested through the construction of fusion proteins containing a fragment of the extracellular domain of the HER2/neu antigen and an antibody Fc γ domain. The resulting fusion protein should target an immunogenic and tumor growth modulatory epitope of HER2/neu to antigen-presenting cells via Fc γ receptors.

INTRODUCTION

The objective of this research project is to test the hypothesis that immune responses to a cancer antigen that is commonly overexpressed in breast cancer can be selectively enhanced and lead to protection against such tumors. This hypothesis of Fc γ receptor-targeted immunization is being tested through the construction of conjugates and fusion proteins containing the HER2/neu antigen and an antibody that targets HER2/neu to antigen-presenting cells via Fc γ receptor IIIA. Appropriate reagents have been prepared and are undergoing testing in murine models. The future plans include the construction of recombinant fusion proteins with similar specificities permits determination of the ability of immunized sera to mediate *in vivo* anti-tumor effects.

We have found that chemical conjugation strategies do not yield reproducible, functional conjugates of anti-Fc γ R antibodies and HER2/neu. Accordingly, we modified the project to target human Fc γ immunoglobulin receptors using a fusion protein consisting of human Immunoglobulin G1 (IgG1)Fc and the HER2/neu extracellular domain (ECD) fragment 3. This fragment contains the epitopes recognized by the clinically used monoclonal antibody, trastuzumab. We believe there is significant potential to induce host antibody responses that will bind to HER2/neu epitopes that perturb receptor signaling. Accordingly, this approach offers the promise of an active immunization strategy that will induce long-lasting host immunity to tumors that overexpress HER2/neu.

BODY

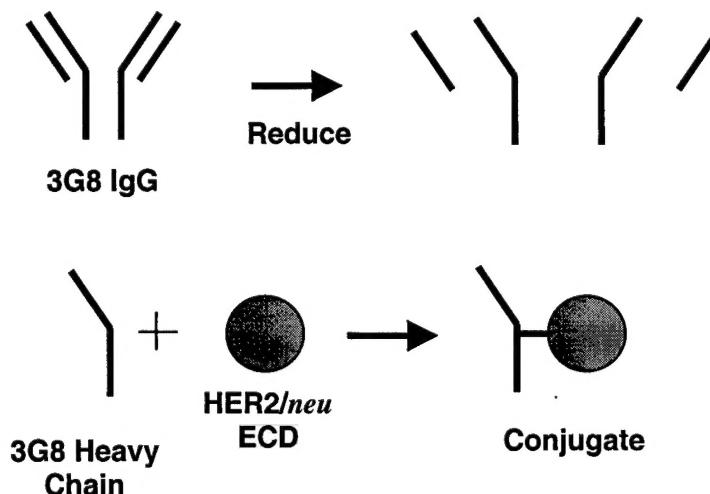
Significant progress has been made in achieving the specific objectives of this research project in the past year. This progress is summarized for each of the major areas of research encompassed by this project.

Creation of a Relevant Animal Model: We have produced a transgenic mouse which expresses the human Fc γ RIIIA (CD16A) and a mutated human HER-2/neu. The HER-2/neu sequence was engineered to contain the Nhe I and Not I restriction sites at the 5' and 3' ends respectively to clone into the pCI mammalian vector under a cytomegalovirus CMV promoter. The HER-2/neu construct was then mutated at position 753, changing lysine to methionine and eradicating the tyrosine kinase activity of the HER-2/neu molecule. In addition, two Bgl II restriction enzyme sites were knocked out by making non-palindromic changes of serine at AA's 614 and 1280, to allow for characterization of the transgene insert in murine genomic DNA. Sequencing confirmed the presence of the mutant codon sequence and elimination of the Bgl II sites. The DNA was purified and microinjected into mouse zygotes and implanted in female transgenic human Fc γ RIIIA mice, reported on in last year's progress report. Forty five transgenic pups were produced. Twenty five pups have had their genomic DNA screened for

evidence of the transgenic insert by PCR analysis; four samples are PCR positive. PCR positive animals have had the transgene insertion confirmed by Southern Blot analysis. Animals with confirmed to have the mutated HER-2/neu transgene are being cross bred.

Production of Recombinant HER2/neu Extracellular Domain Fragments: The anti-HER-2/neu antibody Herceptin has been shown to bind to the region of the HER-2/neu extracellular domain located closest to the transmembrane domain of the molecule. We have generated three fragments that encode the HER-2/neu extracellular domain to determine the epitopes on the extracellular domain of HER-2/neu recognized by the antibodies generated by our immunization strategy. In addition we wish to utilize the region of HER-2/neu demonstrated to have biologic activity in humans in our immunization strategy. Utilizing protein modeling comparisons with the known structure of insulin-like growth factor receptor 1, three fragments containing amino acids 1-185 (ECD F1), 186-339 (ECD F2), and 340-508 (ECD F3) were selected based on their predicted structure and folding characteristics to maximize *in vitro* production. Transient transfection in COS cells, inserting the sequences in the pSec Tag2/Hygro vector revealed abundant secretion of F3, but not of F1 and F2, which remained inside the cells. A stable F3-transfected 293 cell line has been established. F3 is now available in sufficient quantities for future ELISA assays and immunizations as described below.

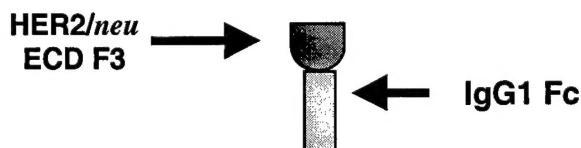
Preparation of HER2/neu ECD – anti-Fc γ RIII Conjugates: Chemical conjugation was chosen as an initial strategy to create reagents to test proof of concept for this



project. . Using standard conjugation kits (Pierce), several methods were explored. The methodology employed oxidation of the 3G8 IgG, attaching a linker, followed by conjugation of reduced HER2/neu ECD with subsequent blockage of unoccupied sulphhydryl groups with glutathione. The resulting conjugate had a molecular weight of 120 kDa (not shown) and possessed both anti-Fc γ RIII and HER2/neu binding activities as

determined by flow cytometry. The proposed structure of the 120kDa conjugate is shown in the preceding figure.

Additional chemical conjugations of whole 3G8 IgG1 and HER2/neu ECD did not produce a product which was at a concentration which allowed adequate purification from unconjugated HER2/neu ECD. It was felt that the low production of conjugated protein was partially due to insufficient glycosylation of the Fc region of the monoclonal anti Fc_γRIII antibodies, 3G8. Therefore, oxidized 3G8 bound minimal linker. An alternate strategy was undertaken to construct a fusion protein consisting of human IgG₁ Fc to HER2/neu ECD fragment 3 (ECD F3).



Future goals will, in addition, produce a fusion protein with the whole ECD portion. Such fusion proteins will appropriately target the immunoglobulin receptor(s) on antigen presenting cells. The fusion protein is currently being characterized for its binding properties. Following characterization, immunization of transgenic C57Bl/6 mice will be performed and assessed as in the previous immunization schema.

Animal Experimentation: The initial immunization experiments have been conducted. Four cohorts of 5 C57Bl/6 human CD16A transgenic mice each were given four subcutaneous immunizations at Day 0, Day 14, Day 28 and Day 56. Equal molar concentrations of each reagent were administered equivalent to 100µg of the conjugate. Retro-orbital eye bleeds were obtained prior to each immunization. On Day 84 mice were terminated. Cardiac bleeds were performed and splenocytes isolated. The cohorts are as follows:

1. HER2/neu ECD
2. 3G8 Heavy Chain – HER2/neu ECD
3. 3G8 Heavy Chain + HER2/neu ECD
4. Phosphate-buffered Saline

The objective of this experiment was to obtain proof-of-concept that targeting the HER2/neu ECD to antigen presenting cells via human Fc_γRIII would induce a more brisk immunization than does immunization with HER2/neu ECD alone. Murine immune responses to HER2/neu ECD were monitored by Enzyme-Linked Immunoabsorbent (ELISA) assays. HER2/neu ECD was employed as the plate coat, plates were blocked and a 1:50 dilution of plasma for all cohorts at each time point was added. Amplification

of the signal was accomplished by adding a goat anti-mouse antibody, followed by a biotinylated anti-goat and streptavidin-horseradish peroxidase. For quantification of antibody (ng/mL) a standard curve was established using a mouse anti-HER2/neu antibody. Antibody responses were detected in all ECD-cohorts.

Antibody in ng/mL	Day 1 (Baseline)	Day 14	Day 28	Day 56	Day 84
PBS control	11	18	9	10	4
HER2/neu ECD alone	37	23,372	13,829	33,110	22,271
3G8 + HER2/neu ECD	7	2936	3947	10,816	5713
3G8- HER2/neu ECD conjugate	0	0	546	748	550

HER-2/neu ECD alone induced a brisk antibody response. When 3G8 was given with, but not conjugated to ECD, 3G8 inhibited the overall antibody response. It is felt that the actual protein concentration of monomeric conjugate of 3G8-ECD was significantly lower than had been determined by spectrophotometric analysis, accounting for the reduced antibody induction by the conjugate.

Splenocytes from the mice were employed in intracellular cytokine assays and analyzed by flow cytometry for the detection of IFN γ following *in vitro* stimulation with HER2/neu ECD to detect the induction of a cellular TH1 response. No cellular responses were detected in these immunized cohorts.

Immunization experiments will be conducted with the newly constructed fusion protein. Plasmas will be analyzed for specificities to the three domains of HER2/neu. Plasma from the first immunization experiment will be reassessed against these three domains to allow for comparisons with results of the fusion protein immunizations. Cellular response will be investigated using the flow cytometric intracellular cytokine assays.

KEY RESEARCH ACCOMPLISHMENTS

- Expression of HER2/neu Extracellular Domain in a Robust Mammalian Expression System

- Development of a strategy to produce conjugates containing equimolar concentrations of HER2/neu ECD and the heavy chain of the 3G8 anti-Fc γ receptor III monoclonal antibody. This conjugate possesses intact binding properties of its constituent components.
- Development and characterization of a mouse strain that is syngeneic with C57Bl/6 and transgenic for human Fc γ RIIIA (CD16A)
- Doubly transgenic C57Bl/6 mice – human CD16A and mutated HER2/neu
- Fusion protein of human IgG₁Fc and HER2/neu ECD F3

REPORTABLE OUTCOMES

1. Amoroso AR, Alpaugh RK, Barth MW, McCall AM, Weiner LM. Production and characterization of mice transgenic for the A and B isoforms of human Fc γ RIII. *Cancer Immunol Immunother* 48:443-455, 1999.
2. Weiner LM. Fc γ Receptor-targeted immunization against HER2/neu. Department of Defense Breast Cancer Research Program Meeting: Era of Hope. June, 2000 (Abstract)
3. Cloning and Expression of: a) HER2/neu Extracellular Domain (ECD)
b) HER2/neu ECD F3 protein
c) HER2/neu ECD F3: Fc fusion protein
4. Development of a mutated human HER2/neu mouse strain.

CONCLUSIONS

Conclusions regarding this project await the conduct of the planned experiments. It has proven difficult to clone and express the necessary reagents to conduct such experimentation, but we have succeeded in producing HER2/neu extracellular domain and in developing reactive and reproducible immunoconjugates with minimal aggregation properties. Finally, a mouse model transgenic for human Fc γ RIII has been developed and fully characterized. Experimental outcomes required modification from the original statement of work in order to define a technically feasible and scientifically valid approach to the central issue of developing new vaccine approaches against HER2/neu.

REFERENCES

Not applicable

Personnel supported by this award:

Louis M. Weiner, MD
Margaret von Mehren, MD
R. Katherine Alpaugh, Ph.D.
Josephine Schultz
Nathaniel Greer